Supplemental Information

Materials and Methods:

All centrifugations were performed at 4 ^{o}C. Samples, buffers, and percoll kept on ice throughout the procedure unless otherwise noted. Centrifugation steps for synaptosome isolation were performed in a swinging bucket rotor (JS13.1) used in a Beckman Avanti J-251 ultracentrifuge (Beckman-Coulter, Brea, CA). Synaptosome loading was performed in a water bath at 37 ^{o}C.

Chemicals were obtained from Sigma-Aldrich unless stated otherwise. Quantum dots and FM4-64 dye were obtained from Invitrogen. Mice were wild type strain C57black6 and used according to animal practices at the University of Washington, courtesy of Sandra Bajjalieh. The procedure developed below was created from published protocols for synaptosome isolation, synaptosome stimulation, and synaptic vesicle isolation. Figure 1 provides a schematic of the process described below.

Synaptosome isolation: Synaptosomes were isolated from adult mice (~21 days old) brains following a protocol adapted from Nagy and Delgado-Escueta (*1*). Isolated brains (approximately 0.4 g each) were homogenized in ice cold homogenization buffer (10 mM HEPES, 300 mM sucrose, pH 7.4) using 10 strokes in a chilled Teflon-glass tissue grinder. The homogenate was centrifuged at 1000× *g* for 10 min, which generated a pellet of nuclear and cellular debris (P1) and a low-speed supernatant (S1). S1 was removed to a clean centrifuge tube and spun at 24500× *g* producing a crude mitochondrial pellet (P2) and supernatant (S2) that was discarded. The crude mitochondrial pellet (P2) was resuspended in cold homogenization buffer for loading onto percoll gradients. Typical concentration of total protein was 6-8 mg/mL.

Preparation of percoll gradient: Percoll gradients were prepared as described elsewhere (*1, 2*). Briefly, percoll was filtered through 100 µM filter prior to use. Two gradients were made consisting of 2 mL of each gradient layer. Gradient consisted of a 23%, 10%, and 3% percoll layer made in sucrose/EDTA buffer (5 mM HEPES, 300 mM sucrose, 1 mM EDTA, pH 7.2). Once the gradients were made, 2 mL of resuspended P2 were overlaid on the 3% percoll layer. The gradients were centrifuged at 22,580× *g* for 20 min with slow acceleration and no braking during the deceleration. Purified synaptosomes were collected at the interface of the 10% and 23% percoll layers. The synaptosome fraction was diluted ~6 fold with Kreb-like buffer (118.5 mM NaCl, 4.7 mM KCl, 1.2 mM $CaCl_2$, 1.18 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, 0.1 mM Na_2HPO_4, pH 7.4) and centrifuged at 18,970× *g* for 10 min to remove any excess percoll. The pellet obtained from this wash step was resuspened in 1 mL Krebs-like buffer and kept on ice until loading was performed. Total protein content was assayed at this point using Bradford assay (Bio-Rad, Hercules,CA). Protein concentration adjusted to ~0.3 mg/mL with Krebs-like buffer before loading.

Quantum dot and FM4-64 loading: Prior to synaptosomal loading, quantum dots were diluted 1:1 in Krebs-like buffer and centrifuged at 10,000× *g* for 10 min to pellet any aggregates that may have formed during storage. FM4-64 dye was made at a stock concentration of 1 mg/mL in distilled water and refrigerated until use. Final concentrations for loading were 200 nM for quantum dots and 20 µM for FM4-64 dye. Before stimulation synaptosomes were incubated for 10 min at 37 ^{o}C with FM4-64 and Q605. Stimulation of synaptosomes was performed for 2 min at 37 ^{o}C with either 30 mM K^+ (high K buffer: 63.2 mM NaCl, 60 mM KCl, 1.2 mM $CaCl_2$, 1.18 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, 0.1 mM Na_2HPO_4, pH 7.4) or 1 mM 4-aminopyridine (4-AP) made in Krebs-like buffer. For experiments labeled as "No K^+", stimulation was performed as stated above except Krebs-like buffer was used as stimulant instead of high K^+ buffer. Experiments labeled "No Ca^{2+}" were performed as stated above but

the reaction buffers did not contain $CaCl_2$. After stimulation, synaptosomes were immediately centrifuged at 8400*x g* for 3 min in an Optima Max tabletop ultracentrifuge (Beckman-Coulter, Brea, CA). Pelleted synaptosomes were washed once with Krebs-like buffer containing 1 mg/mL BSA. A second wash step was performed with Krebs-like buffer without BSA. After final wash step, synaptosomes were re-suspended in ice-cold homogenization buffer.

Synaptic vesicle isolation: Nine volumes of distilled water were added to re-suspended synaptosomes followed by homogenization using 3 strokes in chilled Teflon-glass tissue grinder. Synaptosomes were placed on ice for 30 min to allow full rupture of nerve terminals. Cellular debris was pelleted by centrifugation at 22,460*x g* for 9 min in tabletop ultracentrifuge. The supernatant was removed to a new centrifugation tube and subjected to centrifugation at 217,410*x g* for 1.5 hours in tabletop ultracentrifuge. Synaptic vesicles were collected from pellet by resuspension in small volumes of potassium acetate buffer (20 mM HEPES, 110 mM potassium acetate, 4 mM $MgSO_4$, 4 mM KCl, pH 7.4). Vesicles were stored on ice for immediate use or at 4 ^{o}C for later use.

Optical imaging: Total internal reflection fluorescence was generated by directing 488 nm light from a 20 mW solid-state laser (Coherent Inc., Santa Clara, CA) off axis, via a polychroic mirror, onto the outer edge of the back aperture of a high numerical aperture objective (1.45NA, Nikon), creating total internal reflection at the coverslip/water interface (SI Figure 1). Molecules on the surface were excited by a ~300 nm thick evanescent field created by the internal reflection of the laser beam. Fluorescence emission was collected through the objective and filtered by a 580/70 nm bandpass filter for the Qdot channel or a 660/45 nm bandpass filter for the FM4-64 channel before being imaged onto a PhotonMax EMCCD camera (Princeton Instruments, Trenton, NJ). To image synaptic vesicles, small volume (~100 µL) wells were created by punching holes in a thin layer of polydimethylsiloxane (PDMS) using aluminum tubing (~5 mm diameter) and irreversibly sealing the PDMS chip to a clean borosilicate glass coverslip by

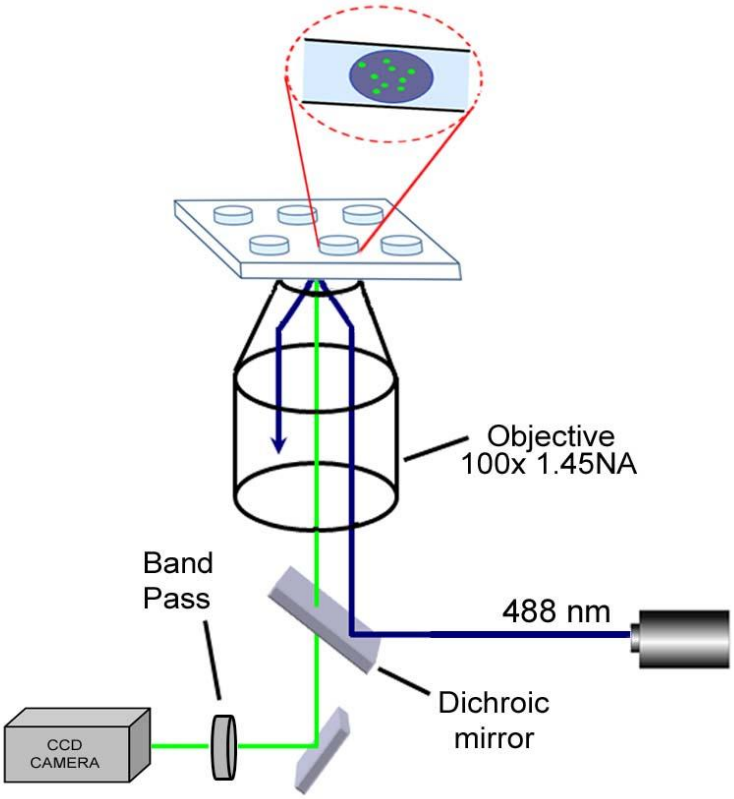
oxidizing the PDMS surface and glass coverslip in oxygen plasma. Before oxidation, the glass coverslip was cleaned thoroughly by boiling for 1 hour in a 3:2:1 mixture of water, ammonium hydroxide, and 30% hydrogen peroxide, followed by thorough rinsing with ultra pure water and drying for 30 min. at 60 ^{o}C. The PDMS was cleaned with a 100% ethanol wash before plasma treatment.

Loaded synaptic vesicles were added to buffer-filled PDMS wells where they immediately sat on the glass surface. When sufficient surface coverage was obtained, unbound or "loose" vesicles were rinsed with three applications of vesicle-free buffer. The fluorescent image for each channel was collected using Metamorph software (Molecular Devices, Silicon Valley, CA). Overlay percentage was determined using an in-house written MATLAB (Mathworks, Natick, MA) program that selected spots based on a Gaussian fit of the fluorescence signal (*3*). Quenching experiments were performed using the same imaging setup and PDMS wells. Black hole quencher-2 amine (BHQ-2 NH_2) was obtained from Biosearch Technologies (Novato, CA) and dissolved into 20 mM HEPES pH 7.4 at a 2 mM stock concentration. BHQ-2 NH_2 was added to PDMS well containing synaptic vesicles at a concentration of 150 µM and allowed to react for 5 min before collecting another image in each channel.

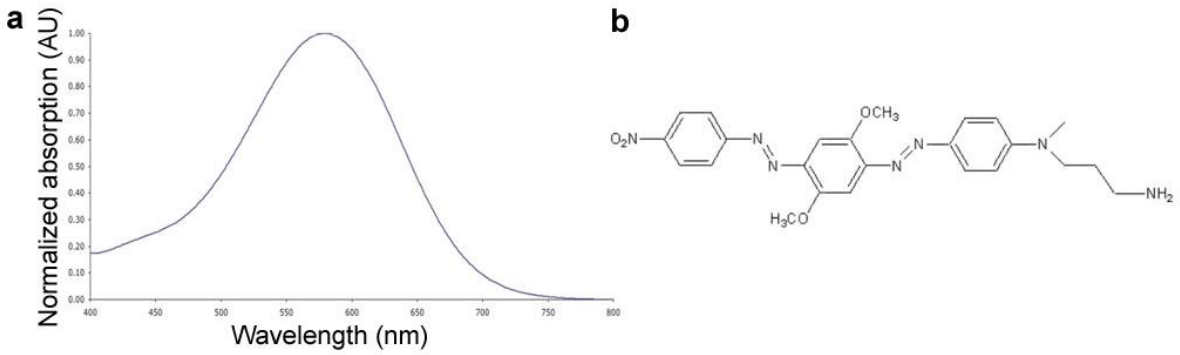
Cryo-electron microscopy imaging: Vesicles were prepared for cryo-electron microscopy as follows. A 3 µL drop of loaded synaptic vesicle sample was applied to a Quantifoil holey carbon grid (Quantifoil, Germany), blotted with filter paper for 7 s at 100 % humidity and immediately frozen in liquid ethane using a Vitrobot (FEI, Hillsboro, Oregon). Grids were mounted onto a pre-cooled Gatan/Oxford CT3500 high-resolution cryo holder, and inserted into an FEI Tecnai F20 microscope equipped with a field emission gun and operated at 200 kv. Images of the vitrified specimen were recorded under low dose conditions at a nominal magnification of 50,000x and defocus values ranging from 1.5 to 3 µm.

References

1. Nagy, A., and Delgadoescueta, A. V. (1984) Rapid Preparation of Synaptosomes from Mammalian Brain Using Nontoxic Isoosmotic Gradient Material (Percoll), *J. Neurochem. 43*, 1114-1123.
2. Dunkley, P. R., Jarvie, P. E., and Robinson, P. J. (2008) A rapid Percoll gradient procedure for preparation of synaptosomes, *Nat. Protoc. 3*, 1718-1728.
3. Mutch, S. A., Kensel-Hammes, P., Gadd, J. C., Fujimoto, B. S., Allen, R. W., Schiro, P. G., Lorenz, R. M., Kuyper, C. L., Kuo, J. S., Bajjalieh, S. M., and Chiu, D. T. (2011) Protein Quantification at the Single Vesicle Level Reveals That a Subset of Synaptic Vesicle Proteins Are Trafficked with High Precision, *J. Neurosci. 31*, 1461-1470.



SI Figure 1: *Imaging setup*. A home-built setup was used to generate total internal reflection fluorescence (TIRF) at the glass/water interface by directing laser light off-axis into the back aperture of a high NA objective. Synaptic vesicles were imaged at the glass surface in PDMS wells. The fluorescent signal was collected by an EMCCD camera.



SI Figure 2: *BHQ-NH_2 structure and spectra.* **a**) Absorption spectra for BHQ®-2 from BioSearch Technologies, Novato, CA. Black-hole quenchers have no native emission. **b**) Structure of BHQ®-2.